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Efficient PCR-based gene targeting in isolates of the nonconventional yeast *Debaryomyces hansenii*

Sondos Alhajouj[1] | Selva Turkolmez[1] | Tarad Abalkhail[1,2] | Zeena Hadi Obaid Alwan[1] | Daniel James Gilmour[1] | Phil J. Mitchell[1] | Ewald H. Hettema[1]

[1]School of Bioscience, University of Sheffield, Sheffield, UK

[2]**Future address:** Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia

Correspondence

Ewald H. Hettema and Phil J. Mitchell, School of Bioscience, University of Sheffield, Sheffield, UK.
Email: E.Hettema@sheffield.ac.uk and P.J.Mitchell@sheffield.ac.uk

Present addresses

Sondos Alhajouj, Department of Biology, College of Science, Jouf University, Sakaka, Saudi Arabia.

Zeena Hadi Obaid Alwan, Department of Biology, College of Science, University of Babylon, Babylon, Iraq.

Abstract

Debaryomyces hansenii is a yeast with considerable biotechnological potential as an osmotolerant, stress-tolerant oleaginous microbe. However, targeted genome modification tools are limited and require a strain with auxotrophic markers. Gene targeting by homologous recombination has been reported to be inefficient, but here we describe a set of reagents and a method that allows gene targeting at high efficiency in wild-type isolates. It uses a simple polymerase chain reaction (PCR)-based amplification that extends a completely heterologous selectable marker with 50 bp flanks identical to the target site in the genome. Transformants integrate the PCR product through homologous recombination at high frequency (>75%). We illustrate the potential of this method by disrupting genes at high efficiency and by expressing a heterologous protein from a safe chromosomal harbour site. These methods should stimulate and facilitate further analysis of *D. hansenii* strains and open the way to engineer strains for biotechnology.

KEYWORDS

Debaryomyces, genome modification, heterologous expression, homologous recombination

1 | INTRODUCTION

The marine ascomycetes yeast *Debaryomyces hansenii* is a nonconventional oleaginous budding yeast. It is found in natural salty environments, and also on salted foods such as cheeses and cured meats, where it contributes to the development of flavour (Prista et al., 2016). Its oleaginous physiology and its resistance to perchlorate to control growth of unwanted microorganisms in bioreactors are considered desirable traits for biotechnology. In addition, interest has grown within the scientific community to use it as a model to understand salt tolerance (Breuer & Harms, 2006; Prista et al., 2016). Most *D. hansenii* strains are haploid that can be diploid temporarily through autogamy (van der Walt et al., 1977). Mating appears to occur very infrequently, but some diploid strains show limited heterozygosity (Jacques et al., 2010; Link et al., 2022). As different isolates display heterogeneity in physiology and genome composition and to unlock and exploit this biological diversity for biotechnology, simple universal synthetic biology tools are essential for further development of *D. hansenii*.

Only a limited number of studies reported the development of research tools including a laboratory strain with auxotrophic markers, a replicating plasmid, gene disruption by homologous recombination

Sondos Alhajouj, Selva Turkolmez and Tarad Abalkhail contributed equally to this study.

using extensive flanking regions, random integration plasmids and CRISPR-Cas9-mediated gene targeting (Biswas et al., 2013; Chawla et al., 2017; Defosse et al., 2018; Minhas et al., 2009, 2012; Spasskaya et al., 2021; Strucko et al., 2021).

Homologous recombination was reported to be inefficient in *D. hansenii* (Minhas et al., 2009, 2012; Spasskaya et al., 2021; Strucko et al., 2021) and long flanking regions were used for targeted integrations (Minhas et al., 2009, 2012). This method relies on multiple plasmid construction steps to generate the gene targeting constructs and the auxotrophic markers *DhHIS4* or *DhARG1* (Biswas et al., 2013; Chawla et al., 2017; Minhas et al., 2009, 2012); therefore, this approach is mostly limited to the single *D. hansenii* strain DH9.

One of the simplest methods of genomic modification is the PCR-mediated gene disruption method developed for gene targeting in *Saccharomyces cerevisiae* (Baudin et al., 1993). In this method, a selectable marker is amplified by PCR, extending the cassette with a 35–50 bp flanking region identical to the target site in the genome on either side. Upon transformation, these short flanking regions are sufficient to direct the selectable marker to the precise site in the genome by homologous recombination. This methodology opened the way for the systematic analysis of each gene and protein in *S. cerevisiae*. For instance, large arrays of gene deletion strains (for an overview see Giaever & Nislow, 2014) and genome-wide libraries in which each open-reading frame (ORF) is tagged with; for instance, GFP (Huh et al., 2003), mCherry (Yofe et al., 2016), or the TAP tag (Gavin et al., 2002) were generated and are available to the yeast research community. In a similar way, gene targeting in *Schizosaccharomyces pombe* and *Kluyveromyces lactis* has been developed (Kaur et al., 1997; Kooistra et al., 2004). Initial studies in *S. cerevisiae* made use of auxotrophic markers. However, since these markers are identical to sequences in the host genome, gene conversions may restore the auxotrophy. Targeting efficiency was increased upon the use of heterologous selectable markers (Bahler et al., 1998; Wach et al., 1994).

Here, we describe an easy method for targeted gene deletion using a PCR-mediated approach as previously developed for *S. cerevisiae*. Regions of 50 nt identity to the target site are sufficient to direct homologous recombination at high efficiency. We developed new selectable marker cassettes existing exclusively of heterologous DNA sequences that confer hygromycin B (HygB) or G418 resistance to *D. hansenii* transformants. Gene disruptions were obtained with high efficiency in two different isolates. Surprisingly, a third isolate showed no phenotypic effects of some of the gene disruptions and a wild-type (WT) copy of the gene was also present, in addition to a disrupted gene copy. As low-copy plasmids that segregate with high fidelity in *D. hansenii* are not available, we used a safe genome harbour site for the integrated expression of heterologous genes in this isolate. With these PCR-based methods and reagents, genetic modification of *D. hansenii* isolates is now economical and easy to achieve and will pave the way to study this yeast in greater depth and develop it for biotechnological uses.

Take-away

- Development of completely heterologous selection markers for *Debaryomyces hansenii*.
- Efficient polymerase chain reaction-based targeted genome modification in *D. hansenii*.
- Safe landing site for heterologous expression.

2 | MATERIALS AND METHODS

2.1 | Strains, growth conditions and media

D. hansenii NCYC102, NCYC3981 and NCYC3363 were obtained from Natural Collection of Yeast Cultures, Norwich Research Park, Norwich, UK (can be accessed via https://www.ncyc.co.uk/). Cells were grown in YMDeb medium containing 0.3% (w/v) yeast extract (Formedium), 0.3% (w/v) malt Eextract (Oxoid), 0.5% (w/v) peptone (Formedium) and 1% (w/v) D-Glucose (Fisher Scientific). For solid medium, 1.5% (w/v) agar (Formedium) was added. Where antibiotic selection was required, HygB (PhytoTech Labs) or G418 disulphate (Melford) was added into the YMDeb medium. Twenty-five or 50 µg/mL HygB was added for selection of NCYC102 or NCYC3363 and NCYC3981 transformants, respectively. Transformants were restreaked on 50–80 µg/mL HygB. G418 disulphate was added at 150 µg/mL for selection of NCYC102 transformants or 350 µg/mL for NCYC3363 transformants. Transformants were restreaked on 500 µg/mL G418. For G418 selection, it was important to plate out at a relatively low cell density to minimise background growth, and to replicate to a fresh selective plate after 24 h incubation.

Yeast minimal arginine-deficient (-ARG) or adenine-deficient (-ADE) medium containing 0.5% (w/v) ammonium sulphate (BDH), 0.19% (w/v) yeast nitrogen base without ammonium sulphate and amino acids (Sigma-Aldrich; Y1251), 2% (w/v) D-glucose, 2% (w/v) agar for solid medium and 0.74 g/L complete supplement mixture (CSM) drop-out: -ARG or -ADE (Formedium).

For fluorescence microscopy, cells were grown to log phase in yeast minimal complete medium containing 0.5% (w/v) ammonium sulphate (BDH), 0.19% (w/v) yeast nitrogen base without ammonium sulphate and amino acids (Sigma-Aldrich; Y1251), 2% (w/v) D-glucose, 1% (w/v) casamino acids (Formedium), 20 µg/mL uracil, 20 µg/mL tryptophan and 30 µg/mL leucine (Sigma-Aldrich).

Escherichia coli DH5α cells were grown at 37°C, either as a liquid culture under shaking at 200 rpm or on solid media, using 2TY media, which contains 1.6% (w/v) tryptone (Formedium), 1% (w/v) yeast extract, 0.5% (w/v) NaCl (Fisher Scientific) and 2% (w/v) agar for solid medium. Where required, ampicillin sodium salt (MP Biomedicals) was added into the medium to a final concentration of 75 µg/mL. The cells were grown at 25°C, either on solid media or in liquid cultures on a shaker at 200 rpm.

TABLE 1 Promoter, terminator and antibiotics resistance ORF sequences that were used in this study.

Insert name	5'→3' DNA sequence
Scheffersomyces stipitis TEF1 promoter region	GGAATGATCCAGAGGCGCGACATTTATGCAGACAATTTGTGTTTTGTCGCAAACGATGTTATAGCGAAATTTTT-CACTCTGTCAGATAAATGGATTTTGTCAAAAGGGGGAAGTAGAAGGAGAATGGGCCCGAGATGTTCTGC-CAAATTCTCAGTAGCATAATGTGAAAGAAGCCCTTACATTGTCCAGCCTCTGGCATCATTAAAAACCGTAGCG-GAAACCAATTGTCTCTGTTCTTCCCTGGCACACCCTGGTAGCCCCATCCAGTTGTAGTACATCTCACACGCTGG-CAACTTGGGACAATCAGCAACTTTTTTTTCTTTTAATTTTTTCAGCGCGACATTTTGCCTCTTCTGCGAGAACA-GACTTTTTCACCTCCATCTCACCCCCCTTTGCACTTATATAAATTGGACCAGTTCCTCCCATTGTA-GAAAAAATTTTGCTGGACCTTTTTCTCTTTTTTTTGTCCTTTAGTTTCATACAATCTAAGTCTATCTACA
S. stipitis TEF1 terminator region	GCTGATTAATTTACGTATATTCAGTTTAATATCAATACGTTAGCTACATTTCCAATGAACGATACTAGATATTGTTTAG-GATTATTGAACTGGTATAGATAATTTTAGTGTATATTCATGTACTTGATAAATGTAATAATATGTGAAAATGTAGTTG-TACATTAACTGATAGACAACATGCTGGAGTATATGGCATTAAGGTTGCTACAAAGTAGAAGCAACCTAGACA-CACCTCAGAAGATAGATTGGG
S. stipitis ACT1 promoter region	AAGTTCCGAGCTTCAGCAAACGCTTGTGTGGAAAGCTCCACCAGTGCTAAGGTGGAGTCGGGTTGGGGAAATGTCGC-GAACGACACAATTTTTCAGCTCAGACGGCACCCCACCAAAAGAATGATAGCAGATAGCCTGGAGAGAGCCCAGAT-CAGCCAAAGAATAGCACTAATATACAAATAATACGAAACCCCAAAATACGACATTGTCCTCCCTTATACACACA-GATGTGGGCTATTTGTGGATGCCAAAATATACCCAATCATGTCGCTATCTAGTGTCTTTTGACTTATCTTCCA-CATTGTTCCCTCTGTGTAGCATGAGCACTCAGCAATGTCGCGTGTCGTGCAAATTTTTCCTTGTGTGCGACTTTCC-CACCCACCGATATTTATAACCAACGCAGTTTTTCTTTTCGTGAGCACAATCCCCTTTTCTTCTTTTTTCAG-TAGGTTTCTGTAATATTAGTACAATCCCTTATATTATAATCATATAGATCAAAC
S. stipitis ACT1 terminator region	AACCACTTGCAAAATCCTTTGTATTCTTGTTCTGCAAACATTTTGTCCAATCTCTTATCTTTCTACGATGTTGAAGAACA-TACATTTTTGTTAGTCCAGCTTGTTATCCTTTTTTTATTAAATAAAAAATTTTATTGT-TAAAGTTGTTTTTCTTCTTTGTCTGTCCTGTTTTAACTCATATATATTCTATATATTACAGAAAGAAAGTAGATCATC-TAAAATAAACATTTATTTGATGAGACAAAGTAGGTTTTTTAGAATACAATGTAGCATCCTCTAATATACAATA-GAGTTGTTTTATAAAAGCGATACATTTAATATTATGACAGGACTGGTGAAGTGTATAGAATTGTGCTAATCTGG-CAAACGAACCCAAGAAGACGTTAAAGCAAATAGTGCA
S. stipitis GPD1 promoter	ATCTGATGTCTCAAATAAGGTATCAAGACAACGAATCAATGATCAACCAAGAAAGATTATTAGAGAAATTGGATGGAT-CAGCTGTGCTTGTGGAAACACTGATACGACATGCCAGTAAACGAAGTAGAAAAAACTAACTGTATATTCGCTAA-CAAAAAAATTGTATTATTTTTAGACATCGTAAATGGGGCTGTAGCCCTAATTATTTTTCATTTCTCGTGATCTG-CACGTGTACTGTTGATTTTTTTGTGCGATTGAAATTATATTGTGCTGTTATCTTAATAAAATCAGTCA-TACCTTTTTTTTTTTGGGTTTTTGTTGTTTAATTTGTGATAAACATCCCATGAGGAACAGCGA-GAAAGTTTTTTGCTGTTCACTTTCTCGTTCAACTTTTGCAAAGTAAAGTAAAGAAAAAAAAATTTTCCCTCGT-CATCTTGATTTTTACTTCTTCTTCTTCTTCTTTTTCCTTTCTTCACACATTATAGTCATAATCAATC
CTG-adapted hygR	ATGGGTAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTAATCGAAAAGTTCGACAGCGTCTCCGACC-TAATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTACGGG-TAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCG-GAAGTGCTTGACATTGGGGAATTTAGCGAGAGCCTAACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTG-CAAGACCTACCTGAAACCGAACTACCCGCTGTTCTACAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCC-GATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTT-CATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCG-CAGGCTCTCGATGAGCTAATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCACGCG-GATTTCGGCTCCAACAATGTCCTAACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGC-GATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCTTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCA-GACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGGCTCCGGGCGTATATGCTCCG-CATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGC-GACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTG-GACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAG-GAATAA
CTG-adapted KanR	ATGGGTAAGGAAAAGACTCACGTTTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGTA-TAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCA-GAGTTGTTTCTAAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGC-TAACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGC-GATCCCCGGCAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTTG-CAGTGTTCCTACGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCT-CAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTT-GAACAAGTCTGGAAAGAAATGCATAAGCTATTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCT-CACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACC-GATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAA-TATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAA

(Continues)

TABLE 1 (Continued)

Insert name	5'→3' DNA sequence
CTG codon-adapted mCherry-PTS1	ATGGTTTCAAAAGGTGAAGAAGATAATATGGCTATTATTAAAGAATTTATGAGATTTAAAGTTCATATGGAAGGTT-CAGTTAATGGTCATGAATTTGAAATTGAAGGTGAAGGTGAAGGTAGACCATATGAAGGTACTCAAACTGCTAAATT-GAAAGTTACTAAAGGTGGTCCATTACCATTTGCTTGGGATATTCTGTCACCACAATTTATGTATGGTTCAAAAGCT-TATGTTAAACATCCAGCTGATATTCCAGATTATTTAAAATTGTCATTTCCAGAAGGTTTTAAATGGGAAAGAGTTAT-GAATTTTGAAGATGGTGGTGTTGTTACTGTTACTCAAGATTCATCATTACAAGATGGTGAATTTATTTATAAAGT-TAAATTGAGAGGTACTAATTTTCCATCAGATGGTCCAGTTATGCAAAAAAAAACTATGGGTTGGGAAGCTTCATCA-GAAAGAATGTATCCAGAAGATGGTGCTTTAAAAGGTGAAATTAAACAAAGATTGAAATTAAAAGATGGTGGTCAT-TATGATGCTGAAGTTAAAACTACTTATAAAGCTAAAAAACCAGTTCAATTACCAGGTGCTTATAATGTTAATAT-TAAATTGGATATTACTTCACATAATGAAGATTATACTATTGTTGAACAATATGAAAGAGCTGAAGGTAGACATT-CAACTGGTGGTATGGATGAATTATATAAACCACTGCACTCAAAACTCTAG

2.2 | Molecular biology techniques

Standard DNA manipulations were performed as described in Sambrook and Russell (2001), except for where kits were used. Plasmids were constructed and amplified in *E. coli* DH5α. Plasmids were purified using QIAGEN Plasmid Miniprep kit, according to manufacturer recommendations. The different PCR polymerases and buffers were supplied by Meridian Bioscience (formerly Bioline). The oligonucleotides were supplied by Merck (formerly Sigma-Aldrich). The PCR protocols were performed as prescribed by the manufacturer. PCR products and plasmid digests were analysed by 0.7% agarose/TAE (Tris-acetate-EDTA) gel electrophoresis. Gels contained 0.5 μg/mL ethidium bromide (Merck) to visualise DNA using a UV transilluminator supplied by GeneSys and captured using GeneSys Software (Geneflow). Molecular weight markers used were the 1 kb HyperLadder (from Bioline). T4 DNA ligase and buffer were purchased from New England Biolabs. The DNA sequences of newly made plasmids were confirmed by Sanger sequencing, which was carried out by Source Bioscience. The samples were prepared and shipped to Source Bioscience Labs using their recommendations. Results were received as a Snapgene file and analysed by the Clustal Omega database (Sievers et al., 2011) by aligning both estimated and obtained plasmid sequences.

2.3 | Generation of selectable cassettes

To generate the HygR marker plasmid, *Klebsiella pneumoniae* HygB phosphotransferase ORF (*hph* ORF) was CTG codon-adapted first, by changing all the CTG codons to other leucine codons. Codon-adapted *hph* ORF was placed in between *TEF1* promoter and terminator from *Scheffersomyces stipitis* (500 bp upstream and 250 bp downstream of *TEF1*, respectively). To generate the KanR cassette plasmid as a selectable marker, the bacterial kanamycin resistance (kanr) ORF from the *E. coli* transposon Tn903, which confers resistance to G418/geneticin in eukaryotes, was CTG codon-adapted first and then placed under control of the *S. stipitis ACT1* promoter and terminator (500 bp upstream and 250 by downstream region of the *ACT1* ORF respectively). For both plasmids, the commonly used restriction sites within the expression cassette sequences were removed in a way that the corresponding amino acid sequences would not change. The selectable marker regions (promoter–antibiotics resistance ORF–terminator sequences) in both plasmids were flanked by multiple cloning sites to allow cloning, as well as *loxP* sites for future further recycling of the plasmids if necessary. Finally, both selectable markers were synthesised artificially and cloned into pUC19 by GenScript. For DNA sequences of various parts, see Table 1.

2.4 | Plasmid construction

ARG1 gene targeting constructs was generated by first amplifying the genomic region upstream of the *ARG1* gene using primers VIP3967 and 3968, which contain *Eco*RI and *Bam*HI restriction site (for all oligonucleotides used in this study, see Table 2). The downstream region was amplified using VIP3969 and 3970, which contain *Sal*I and *Sph*I restriction sites, respectively. The PCR products were cloned into pHygR and pKanR using the restriction sites mentioned above. Peroxisomal marker plasmid was constructed as follows.

The plasmids pAYCU257 and pAYCU272 were a gift from Defosse et al. (2018). Both plasmids were double digested with *Bam*HI and *Sal*I. Subsequently, the *Streptococcus thermophilus* lacZ in pAYCU272 was replaced with yemCherry from pAYCU257, which gave rise to pDEB12. Using pDEB12 as a template, the *Meyerozyma guilliermondii ACT1* promoter–CTG codon-adapted yemCherry ORF sequence was amplified using primers VIP4780 and VIP4781, the latter adds the peroxisomal targeting signal type 1 (PTS1)-P-L-H-S-K-L to the 3′ end of the CTG codon-adapted mCherry. The resulting product was cloned into pSA4 (pKanR flanked by the long *DhARG1* homology arms) in between *Not*I and *Sal*I sites, which yielded pSLV35 plasmid. The *S. stipitis GPD1* promoter (500 bp upstream of *GPD1* ORF) was synthesised by GenScript and provided in pUC19. Using the oligonucleotides VIP4798 and VIP4799, the *S. stipitis GPD1* promoter was amplified by PCR to replace the *M. guilliermondii ACT1* promoter in pSLV35 by cloning, using *Not*I and *Pst*I sites. This yielded pSLV37. For plasmid design and DNA sequences of various parts see Table 1. All plasmids used in this study are summarised in Table 3.

TABLE 2 Oligonucleotides were used in this study.

Oligo name	5′--> 3′ sequence	Description/application
VIP49	GTTTTCCCAGTCACGACG	In all the *Escherichia coli* plasmids. To be used for sequencing, colony PCR and linearization of the gene deletion cassettes where necessary
VIP50	GGAAACAGCTATGACCATG	
VIP4065	TTACCCGTAGGACATATCCACG	Forward and reverse primers within the *hph* ORF. To check the integration of HygB^r marker into the genome
VIP4066	CTATCAGAGCTTGGTTGACG	
VIP3967	CAATGAATTCCCTGTAGTTGTAGATGCCAC	Forward and reverse primers to clone 916 bp upstream of *DhARG1* into pHygR or pKanR as *Eco*RI–*Bam*HI fragment
VIP3968	CAATGGATCCGGCAATAGTGATCGGATTG	
VIP3969	CAATGTCGACTAATCAGCAGTCCAGTACTC	Forward and reverse primers to clone 947 bp downstream of *DhARG1* into pHygR or pKanR as *Sal*I–*Sph*I fragment
VIP3970	CAATGCATGCATGGGGACAAGTTGGCTAGATG	
VIP4016	AGGAGCGCGGTATATAGATC	Forward and reverse primers that anneal outside 900 bp upstream and downstream flanks of *DhARG1*, respectively
VIP4019	CAGCGGGTATAGTTGGAATG	
VIP4161	CCTTCTTCCAATAACCAAGC	Forward and reverse primers that anneal inside the *DhARG1* ORF
VIP4018	GTCCGTGCCATTAGAGATCA	
VIP4262	CAAGACTATGGCACTTGATG	Forward and reverse primers that anneal to *DhARG1* gene deletion construct to decrease the size of the homology flanks to 185 and 228 bp, respectively
VIP4263	CGATGAAGGTGAAAGTTTGC	
VIP4260	GGTAATCATACACATAAAGG	Forward and reverse primers that anneal to *DhARG1* gene deletion construct to decrease the size of the homology flanks to 111 and 163 bp, respectively
VIP4261	CTGATGGCCAATGTAATATC	
VIP4258	TTCCTGTGAACCTGGCTCAG	Forward and reverse primers that anneal to *DhARG1* gene deletion construct to decrease the size of the homology flanks to 73 and 116 bp, respectively.
VIP4259	TACAGACGTCTTGATGAATC	
VIP4298	GAGCGCAAAGCGAGCTTGTA	Forward and reverse primers that anneal to *DhARG1* gene deletion construct to decrease the size of the homology flanks to 43 and 63 bp, respectively.
VIP4299	GTACGATTTTGATTATACCG	
VIP4383	GAGCTTGTAAGAACAATCCGATCACTATTGCCGGTTT-CATGTTGAATTGAGTGAATTCGAGCTCGGTACC	Forward primer that anneals on pHygR to introduce 50 bp upstream of *DhARG1* locus
VIP4384	TATACCGTATTCGACGATGAATACGAAAATGAGTACTG-GACTGCTGATTAAAGCTTGCATGCCTGCAGGTC	Reverse primer that anneals on pHygR to introduce 50 bp downstream of *DhARG1* locus
VIP4489	ATAGAATTAGTGTTATTTTACGTTAATCAGTTACATATTAT-TATAAAAGAGAATTCGAGCTCGGTACC	Forward and reverse primers that anneal on pHygR or pKanR and introduce 50 bp upstream and downstream of *DhARG1* ORF, respectively
VIP4490	TATTTTAGATTCCCTATTTAATTATCTTTATAGTTAGTC-GAGTGCAACAAAGCTTGCATGCCTGCAGGTC	
VIP4385	CAGGCGGACGACGGGGCTA	Reverse and forward primers to check integration of HygR in *DhADE2* gene
VIP4388	GACGGTATCGAAGGATAGC	
VIP4780	CATGCGGCCGCACCCGCTCTTGACGGTTAC	To amplify *MgACT1* promoter–CTG codon-optimised mCherry from pDEB12. VIP4781 introduces P-L-H-S-K-L to the end of mCherry for peroxisomal marker
VIP4781	CAGGTCGACCTAGAGTTTTGAGTGCAGTGGTTTATA-TAATTCATCCATACCACC	
VIP4798	CATGCGGCCGCCGAAGTTATATCTGATGTCTC	To amplify *SsGPD1* promoter and clone it into pSLV35 to replace *MgACT1* promoter
VIP4799	CAGCTGCAGGATTGATTATGACTATAATGTGTG	
VIP4793	GAAGATGGTGGTGTTGTTAC	Within mCherry. To check the integration of mCherry-PTS1 into *DhARG1* locus
VIP4342	CTTCATTACACGAAATCGAAATAGCACGTTTATTCATATA-TAGAGAGACGGAATGATCCAGAGGC	Forward primer that anneals on pHygR to introduce 50 bp upstream and downstream of *DhADE2* ORF
VIP4343	TCTATCTAATACTAGAAATAAGTATAAAGCAAACTCTA-TAACAACCGAATCCCAATCTATCTTCTGAGG	Reverse primer that anneals on pHygR to introduce 50 bp upstream and downstream of *DhADE2* ORF

(Continues)

TABLE 2 (Continued)

Oligo name	5′--> 3′ sequence	Description/application
VIP4372	TTATAAGATAAAGTACTTCATTACACGAAATCG	Forward primer that anneals on pHygR flanked by 50 bp upstream and downstream of *DhADE2* ORF to generate 65 bp flanks
VIP4373	ATTGCTTGGATGGTTTTTTCTATCTAATACTAGAAATAAG-TATAAAGCAAACTCTATAACAACCG	Reverse primer that anneals on pHygR flanked by 50 bp upstream and downstream of *DhADE2* ORF to generate 65 bp flanks
VIP4375	TAGTTTATTATTATAAGATAAAGTACTTCATTACAC-GAAATCG	Forward primer that anneals on pHygR flanked by 50 bp upstream and downstream of *DhADE2* ORF to generate 75 bp flanks
VIP4376	TGGATTAAATATTGCTTGGATGGTTTTTTCTATCTAATAC-TAGAAATAAGTATAAAGCAAACTCTATAACAACCG	Reverse primer that anneals on pHygR flanked by 50 bp upstream and downstream of *DhADE2* ORF to generate 75 bp flanks

Abbreviations: HygB, hygromycin B; ORF, open-reading frame.

TABLE 3 List of plasmids used in this study.

Plasmid name	Insert	Parental vector	Purpose	Source
pHygR	*loxP*-HygR expression marker (*Scheffersomyces stipitis TEF1* promoter–CTG-adapted hygR ORF–*S. stipitis TEF1* terminator)-*loxP*	pUC19	To generate gene deletions/modifications in *Debaryomyces hansenii* using HygR as a marker	GenScript
pKanR	*loxP*-KanR expression marker (*S. stipitis ACT1* promoter–CTG adapted KanR ORF–*S. stipitis ACT1* terminator)-*loxP*	pUC19	To generate gene deletions/modifications in *D. hansenii* using KanR as a marker	GenScript
pSA4	~1 kb of an upstream flank of *DhARG1* *Eco*R1–*Bam*H1 fragment ~1 kb of a downstream flank of *DhARG1* *Sal*1–*Sph*1 fragment	pKanR	Used as a backbone plasmid to generate peroxisomal marker plasmid with mCherry-PTS1	This study
pSA5	~0.9 kb of an upstream flank of *DhARG1* *Eco*R1–BamH1 fragment ~1 kb of a downstream flank of *DhARG1* *Sal*1–*Sph*1 fragment	pHygR	To generate *DhARG1* deletion cassette using long flanks of *DhARG1*	This study
pDEB12	yemCherry from pAYCU257 (Defosse et al., 2018) *Bam*H1–*Sal*1 fragment	pAYCU272 (Defosse et al., 2018)	To swop the *StlacZ* in pAYCU272 with yemCherry of pAYCU257, after digesting both vectors with *BamH*1 and *Sal*1	Defosse et al. (2018)
pSLV35	*MgACT1* promoter-CTG codon-adapted yemCherry- PTS1 *Not*1–*Sal*1 fragment	pSA4	To generate red peroxisomal marker construct plasmid backbone	This study
pSLV37	*S. stipitis GPD1* promoter *Not*1–*Pst*1 fragment	pSLV35	To generate final peroxisomal marker construct behind *SsGPD1* promoter	This study

Abbreviations: HygB, hygromycin B; ORF, open-reading frame.

2.5 | Transformation of PCR products

Electroporation was based on a previously described method by (Minhas et al., 2009) with some small adaptations (Alwan, 2017). A fresh culture was diluted in 50 mL of YM medium at OD_{600} = 0.025–0.0125 and grown overnight. The next day, when cells reached OD_{600} = 2.6–2.8, the cells were transferred into a sterile 50 mL screw cap tube and centrifuged for 5 min at 1700*g*. The supernatant was discarded, and the cell pellet was resuspended in 1 mL of 50 mM sodium phosphate buffer, pH 7.5, containing 25 mM DTT. Cells were then incubated at 25°C for 15 min and centrifuged for 5 min at 1700*g*. The cell pellet was resuspended in 8 mL sterile water (4°C) and centrifuged as described previously. The supernatant was discarded, the cell pellet was resuspended in 1 mL sterile ice-cold 1 M sorbitol and centrifuged as before. The supernatant was discarded, and the cells were resuspended in the remaining liquid to obtain a dense suspension (ca. 200 µL). Of the cell suspension, 40 µL was transferred to a microfuge tube and

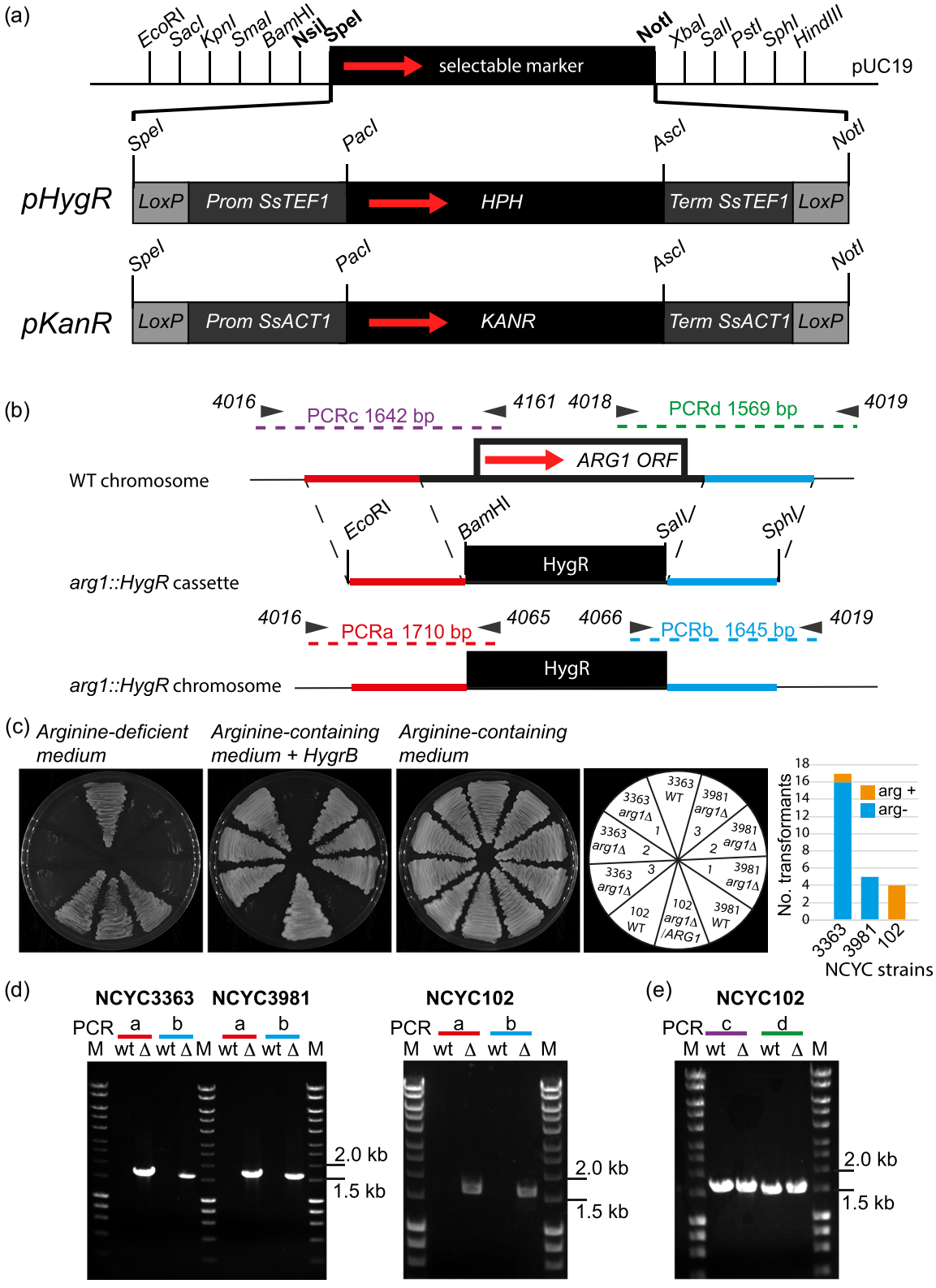


FIGURE 1 (See caption on next page).

mixed with PCR product. Initial experiments used PCR product directly, but during this study, it became clear that 500 ng EtOH-precipitated PCR product dissolved in 1–2 µL ultrapure water resulted in a consistently higher number of transformants. The mixture was placed in a precooled 2 mm electroporation cuvette (Geneflow) and incubated on ice for 5 min. Electroporation was performed with an Equibio electroporator (a BioRad Gene Pulser) at 2.3 kV, 1000 Ω and 50 µF with a pulse as exponential decay. Following electroporation, the cells were resuspended with 1 mL YM medium containing 0.1 M sorbitol. The cell suspension was transferred to a 2 mL microfuge tube. The samples were incubated for 4 h at 25°C with shaking at 200 rpm. The cells were spread onto YM plates containing the appropriate antibiotic and incubated at 25°C. After 3 days, single colonies were picked and restreaked on appropriate selective media.

2.6 | Yeast total DNA isolation

Cells from a 3 mL overnight culture were harvested in a 2 mL screw cap tube (by centrifuging at 10,625 rcf for 1 min). The resultant pellets were washed with 1 mL sterile H_2O. The pellets were resuspended in 200 µL TENTS solution (1% sodium dodecyl sulphate [w/v], 2% Triton X-100 [v/v], 1 mM EDTA, 100 mM NaCl and 20 mM Tris-HCl [pH 8]), 200 µL phenol–chloroform–isoamyl alcohol (25:24:1) and 200 µL 425–600 µm glass beads (BioSpec Products) were added into each tube. The tubes were placed into the bead beater (BioSpec Products) at maximum speed for 45 s, then centrifuged for 30 s at 10,625 rcf. Another 200 µL TENTS solution was added into each tube followed by a brief vortex, the tubes were centrifuged at 10,625 rcf for 5 min. The supernatants were transferred into Eppendorf tubes and another 200 µL phenol–chloroform–isoamyl alcohol was added to each tube. Each tube was vortexed and centrifuged at 10,625 rcf for 5 min. The supernatants (~300 µL) were transferred to new tubes. Each supernatant was mixed with 1/10th volume of 3 M NaAc (pH 5.2) and 2.5× volume 100% EtOH, and the tubes were kept on ice for 15 min. Each tube was centrifuged at 4°C at 12,000 rpm for 15 min, using the accuSpin Micro R centrifuge (Fisher Scientific). The supernatant was removed and the pellets were washed with 300–500 µL 70% EtOH and centrifuged in Sigma 1-14 microfuge at maximum speed for 5 min. The supernatant was removed and each pellet was resuspended with 200 µL 1× TE (at pH 7.4) + 2 µL RNAse (10 mg/mL), incubated at room temperature for 10 min. Then, they were mixed with 1/10th volume of 3 M NaAc (pH 5.2) and 2.5× volume 100% EtOH and kept on ice for 15–30 min. Each tube was centrifuged at 4°C and 12,000 rpm for 15 min, using the accuSpin Micro R centrifuge (Fisher Scientific). The pellets were washed with 300–500 µL 70% EtOH and centrifuged in Sigma 1-14 microfuge at maximum speed for 5 min. After the supernatants were discarded, the pellets were air-dried. Finally, each pellet was resuspended in 50 µL TE (pH 7.4) and stored at −20°C.

2.7 | Fluorescence microscopy

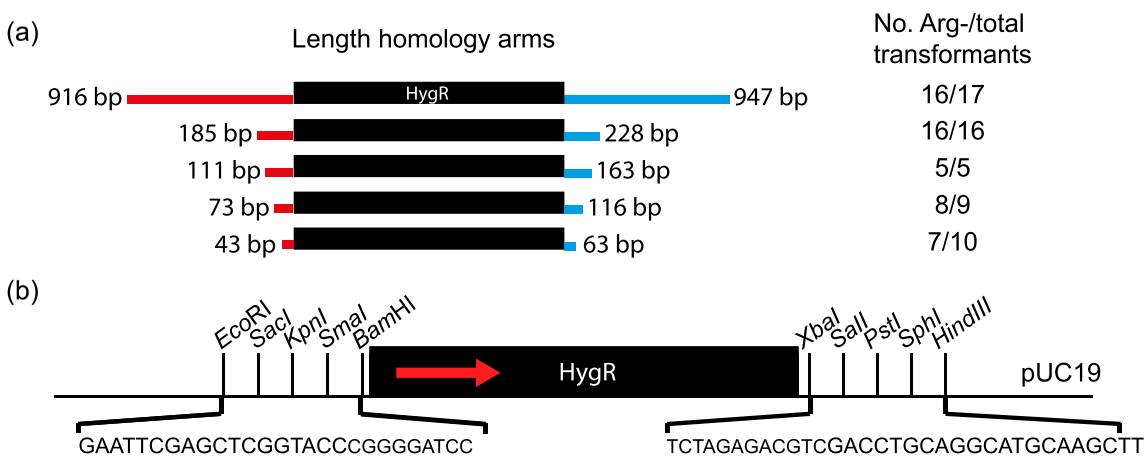
Fluorescence microscopy Live cell imaging of *D. hansenii* cells was performed as described previously for *S. cerevisiae* cells (Ekal et al., 2023). Basically, cells were grown to log phase in minimal 2% glucose medium and analysed with a microscope (Axiovert 200 M; Carl Zeiss) equipped with an Exfo X-cite 120 excitation light source, bandpass filters (Carl Zeiss and Chroma Technology), a Plan-Apochromat ×63 1.4 NA objective lens (Carl Zeiss) and a digital camera (Orca ER; Hamamatsu Photonics). Image acquisition was performed using Volocity software (PerkinElmer). Fluorescence images were collected as 0.5 µm *z*-stacks, merged into one plane using Openlab software (PerkinElmer) and processed further in Photoshop (Adobe). Bright-field images were collected in one plane and processed where necessary to highlight the circumference of the cells in blue.

3 | RESULTS AND DISCUSSION

3.1 | Development of new dominant selectable marker cassettes for *D. hansenii*

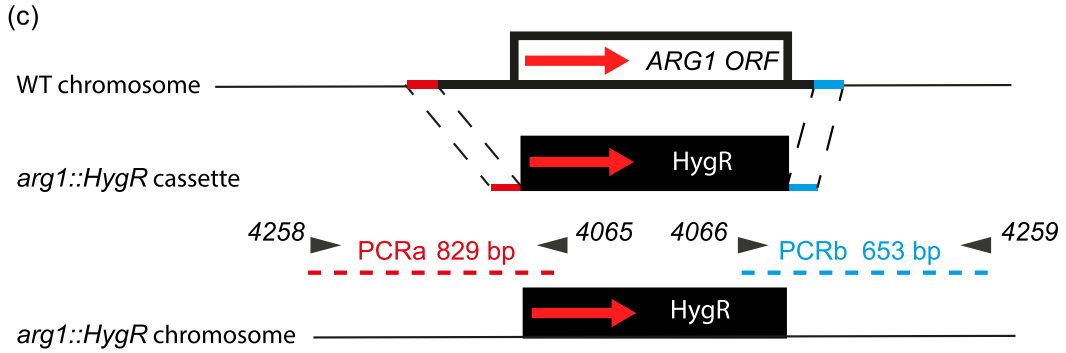
We set out to improve the efficiency of HR-mediated genome editing by generating a completely heterologous marker to allow for genome editing in WT isolates of *D. hansenii* and to reduce the background of gene conversions by auxotrophic mutations. *D. hansenii* is a member of the CTG clade of yeasts, which also includes *Candida albicans* and

FIGURE 1 *ARG1* gene deletion strategy using completely heterologous antibiotic resistance marker cassettes. (a) Schematic of antibiotic selection cassettes in pUC19 for use in *Debaryomyces hansenii*, various elements not to scale. Restriction sites used for cloning homology arms and for identifying insert direction are indicated. Grey boxes represent the promoter (Prom) and terminator (Term) sequences of the *Scheffersomyces stipitis* translation elongation factor 1 gene or Actin 1 gene. Black boxes contain the *hph* gene from *Klebsiella pneumoniae* or the *kanr* gene from *E. coli*. pHygR and pKanR, plasmids encoding the new cassettes. (b) *ARG1* gene deletion strategy using 0.9–1 kb homology flanks. Black arrows containing numbers represent primers and the expected size of PCR products is indicated. Red and blue lines indicate flanking regions of the *ARG1* gene locus cloned into pHygR using restriction enzyme sites indicated. Red arrow, direction of transcription (a, b). (c) Growth analysis of a selection of yeast strains and transformants as indicated on minimal arginine-deficient medium and arginine-containing medium, and arginine-containing medium containing 80 µg/mL HygrB. (d, e) Agarose gel electrophoresis analysis of analytical PCR a, b, c and d as indicated in (b) on total DNA of wild-type cells (WT) or hygromycin B-resistant colonies (Δ). M, molecular weight marker.



Forward gene deletion primer: 5'- (50 nt gene-specific sequence)-GAATTCGAGCTCGGTACC-3'

Reverse gene deletion primer: 5'- (50 nt gene-specific sequence)-AAGCTTGCATGCCTGCAGGTC-3'



(d)

No. arg-/total transformants

strain	Ethanol precipitation	Purification kit
NCYC 3363	16/22	3/3
NCYC 3363	16/22	12/14
NCYC 3363	16/20	2/3
NCYC 3363	13/19	
NCYC 3363	16/17	
NCYC 3363	22/25	
NCYC3981	9/10	
NCYC3981	16/21	
NCYC3981	10/12	

(e)

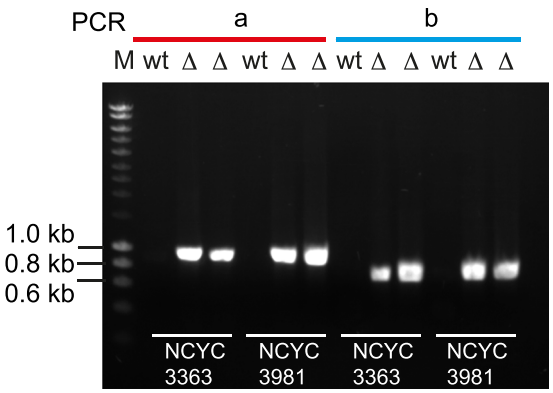


FIGURE 2 (See caption on next page).

S. stipitis, wherein CUG codons, a common codon for leucine, is read as serine (Sugita & Nakase, 1999). We designed new selectable markers with the design of the hphMX and kanMX cassette for *S. cerevisiae* gene targeting in mind (see Figure 1a) (Goldstein & McCusker, 1999; Guldener, 1996; Wach et al., 1994). The CTG codons of the *K. pneumoniae hph* ORF, encoded by the *hph* gene (Gritz & Davies, 1983), were replaced by other leucine codons (for DNA sequence see Table 1). To allow the efficient expression of hph, a strong heterologous promoter (500 bp upstream region of the *S. stipitis TEF1* ORF) and terminator (250 bp downstream region of the *S. stipitis TEF1* ORF) sequences were selected. This marker cassette is flanked by *loxP* sites for future recycling. Additional restriction sites were introduced for convenience of cloning. The complete hygromycin B resistance cassette (HygR) was synthesised in pUC19 (GenScript) (Figure 1a) and called pHygR. The KanR cassette was designed in a similar way. The bacterial kanr ORF from the *E. coli* transposon Tn903, which confers resistance to G418/geneticin in eukaryotes, was placed under control of the *S. stipitis ACT1* promoter (500 bp upstream region of the *ACT1* ORF) and terminator (250 bp downstream of the *ACT1* ORF). The cassette is flanked by *loxP* sites that are surrounded by additional restriction sites. The CTG codons in the kanr ORF were replaced by CTA codons. The complete cassette (KanR) was synthesised in pUC19 (GenScript; for DNA sequence see Table 1) and called pKanR (Figure 1a).

To test the efficiency of the new HygR cassette in targeted gene deletions, we flanked the *hph* cassette with approximately 900 bp– 1kb upstream and downstream regions of the arginine–succinate synthase encoding gene *ARG1* (Figure 1b) and transformed this through electroporation into the three different natural *D. hansenii* isolates NCYC102, NCYC3363 and NCYC3981. Transformants were selected on 25 μg/mL hygromycin B (for NCYC102) or 50 μg/mL hygromycin B (for NCYC3981 and NCYC3363) and transformants were readily obtained. Hygromycin B-resistant colonies were restreaked and subsequently tested for arginine auxotrophy. From the five and 17 NCYC3981 and NCYC3363 transformants obtained, five and 16 required arginine for growth, respectively (Figure 1c). This suggested a high efficiency of targeted gene deletion in these two isolates. Correct integration was confirmed by analytical PCR (Figure 1d). Surprisingly, none of the four NCYC102 transformants required arginine for growth (Figure 1c), although the HygR cassette had integrated properly into the *ARG1* locus in all four of the colonies tested (one shown in Figure 1d). Further PCR-based analysis revealed the presence of an additional copy of *ARG1* in NCYC102 *arg1::HygR* transformants (Figure 1e).

3.2 | Short homology arms direct efficient gene disruption

To test whether short homology arms promote homologous recombination in *D. hansenii*, oligonucleotides were designed to anneal on the original *ARG1* knockout (KO) cassette to achieve shorter flanks surrounding the *HygR* selection marker (see Figure 2a). The differently sized *ARG1* deletion cassettes were amplified by PCR and were electroporated into NCYC3363. Transformants were selected on 50 μg/mL hygromycin B. All PCR-generated *ARG1* KO cassettes yielded transformants with the majority of transformants requiring arginine for growth (Figure 2a). As homology arms of 43–63 bp still directed targeted deletion of the *ARG1* locus, we next designed oligonucleotides that directly annealed onto pHygR and contained 50 bp 5′ extensions identical to the DNA sequence of the *ARG1* locus according to the CBS767 genome sequence (Figure 2b,c). Using these oligonucleotides, the deletion cassette was amplified by PCR and was electroporated into NCYC3363 cells. We noticed variability between the transformation efficiency of different PCR preparations and tested different PCR purification protocols. We found that electroporation of 500 ng of ethanol-precipitated PCR product resulted in 17–25 transformants per transformation, whereas electroporation of 500 ng PCR products cleaned up by a commercial PCR clean-up kit resulted in a lower number of transformants (Figure 2d). Subsequently, we also transformed NCYC3981 using 500 ng ethanol-precipitated PCR product and obtained between 10 and 21 transformants. (Figure 2d). These conditions were used for further experiments (see also Section 2 for details). When we combine the results of these experiments, 106 of the 145 NCYC3363 transformants (73%) and 38 of the 47 NCYC3981 transformants (80%) failed to grow on an arginine-deficient medium. We confirmed correct integration of the *HygR* cassette into the *ARG1* locus in two randomly selected arginine auxotrophs of each strain background (Figure 2e). We conclude that PCR-mediated gene disruption is a very efficient way of generating mutants in *D. hansenii* NCYC3363 and NCYC3891 cells.

To test whether PCR-based gene deletion was also efficient at other loci, we tested the *ADE2* locus. Three different *ADE2* KO cassettes were produced through PCR on pHygR. The cassettes

FIGURE 2 Short homology arms direct gene targeting with high efficiency. (a) Schematic showing the reduction of homology arm length of the *ARG1* knockout construct and the resulting number of arginine auxotrophs (-ARG) among the total hygromycin B-resistant transformants in the strain NCYC3363. (b) Schematic showing design of primers to amplify the completely heterologous antibiotic resistance cassettes for gene deletions using 50 bp homology arms. (c) *ARG1* gene deletion strategy using 50 bp homology flanks generated by direct PCR on pHygR. Red arrow, direction of transcription. Black arrows containing numbers represent primers and the expected size of PCR products is indicated. Red and blue lines indicate 50 bp flanking regions just upstream and downstream of the *ARG1* ORF. (d) Table of transformant frequency and arginine auxotrophy during tests of PCR product purification. (e) Agarose gel electrophoresis analysis of PCR a and b products as indicated in (c) on total DNA of wild-type cells (WT) or two hygromycin B-resistant colonies (Δ) in the strains NCYC3363 and NCYC3891. M, molecular weight marker.

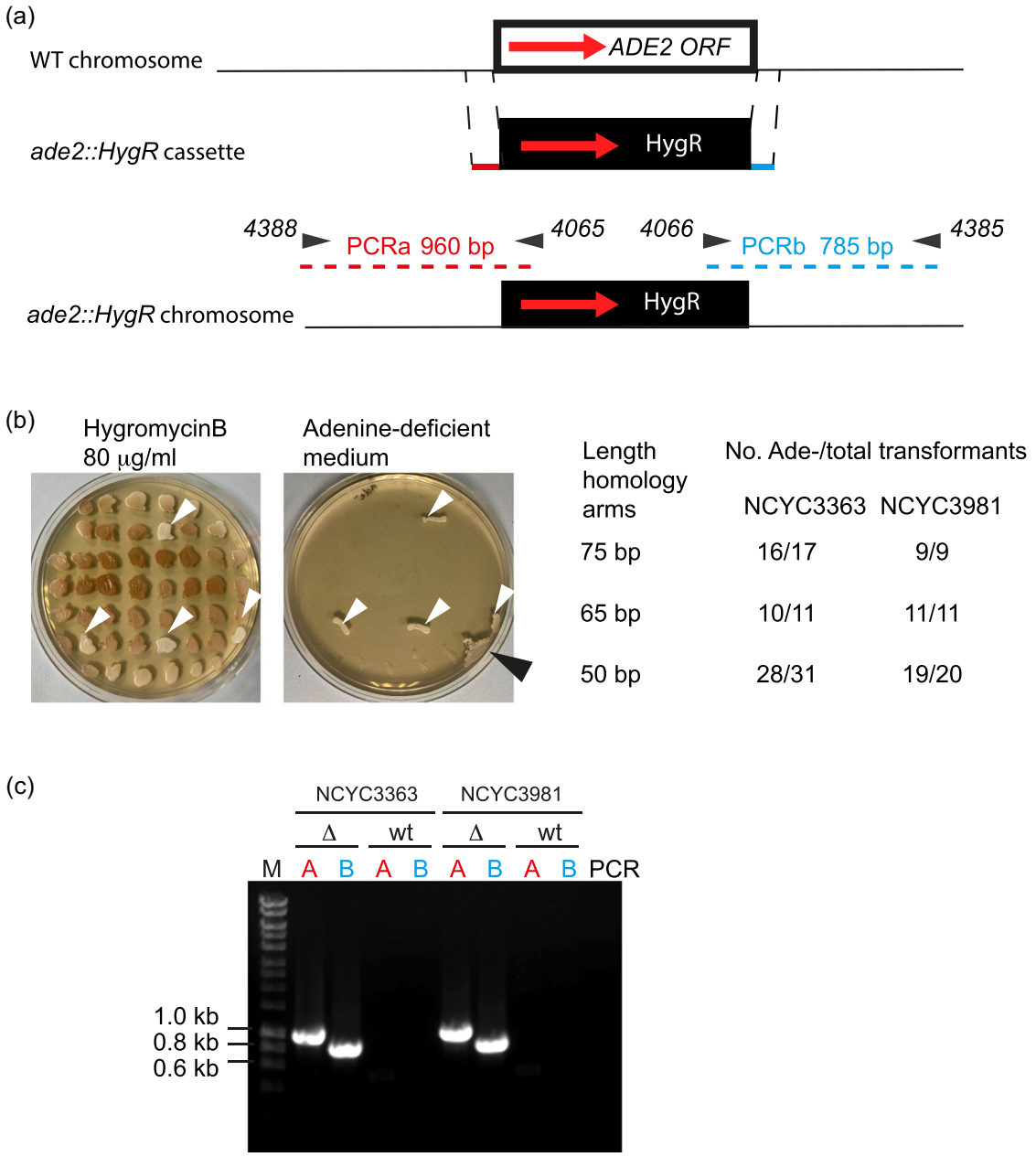


FIGURE 3 *ADE2* gene deletion using 50, 65 and 75 bp homology flanks generated by direct PCR on pHygR. Red arrow, direction of transcription. Black arrows containing numbers represent primers and the expected size of PCR products is indicated. Red and blue lines indicate 50 bp flanking regions just upstream and downstream of the *ADE2* ORF. (b) Growth analysis of a collection of 49 NCYC3361 transformants patched onto hygromycin B-containing medium, grown for 2 days at 25°C and from there they were patched on minimal adenine-deficient (-ADE) medium and incubated for another 2 days at 25°C. Note the white patches on the hygromycin B medium correspond to adenine prototrophs as indicated by white arrowheads. Black arrowhead indicates a patch of untransformed WT cells added as positive control onto -ADE plates, as these cells do not grow on hygromycin B plate. The resulting number of adenine auxotrophs (-ADE) among the total hygromycin B-resistant transformants in the strain NCYC3363 NCYC3981 is indicated. (c) Agarose gel electrophoresis analysis of PCR a and b products as indicated in (a) on total DNA of wild-type cells (WT) or hygromycin B-resistant colonies (Δ) in the strains NCYC3363 and NCYC3891. M, molecular weight marker.

varied in the length of homology arms (75, 65 and 50 bp) identical to the regions directly upstream and downstream of the *ADE2* ORF (Figure 3a). The PCR products were ethanol precipitated and 500 ng electroporated into NCYC3363 and NCYC3981 cells, and transformants were selected on hygromycin B-containing media. Colonies were then streaked onto hygromycin B-containing medium and grown for 2 days before restreaking onto -ADE plates (Figure 3b). The vast majority of transformants (>90%) in NCYC3363 and NCYC3981 failed to grow on the -ADE medium (Figure 3b). Adenine auxotrophs accumulate a brown, reddish intermediate in the adenine

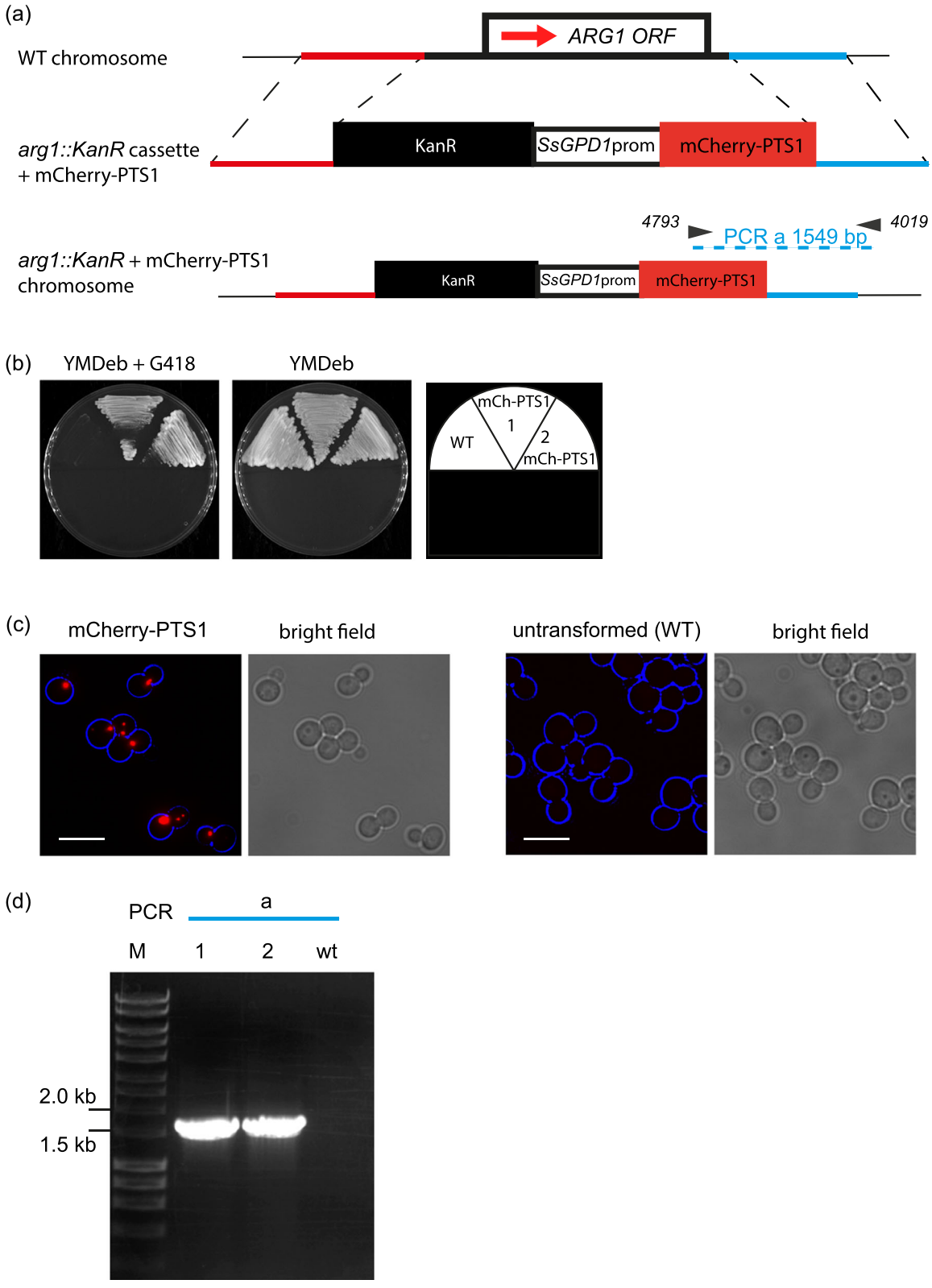


FIGURE 4 (See caption on next page).

biosynthetic pathway, hence the colour difference between adenine auxotrophs and prototrophs (Figure 3b). PCR analysis confirmed correct integration of the *HygR* cassette into the *ADE2* locus in a randomly selected adenine auxotroph of each strain background (Figure 3c). We conclude that gene targeting occurs efficiently at both the *ARG1* and the *ADE2* locus using short regions of homology.

3.3 | Safe harbour site for heterologous expression in the *ARG1* locus

As low-copy plasmids that segregate with high fidelity in *D. hansenii* are not available, and since random integration of expression cassettes in the genome may affect normal cell function and result in various levels of expression, we explored the expression of heterologous proteins by insertion of expression cassettes into one of the *ARG1* loci of NCYC102. As a test, we generated a universal peroxisomal marker by appending the red fluorescent protein mCherry with a PTS1. Peroxisomes are small organelles that posttranslationally import proteins into their lumen and that are gaining interest in synthetic biology circles to house new biosynthetic pathways for metabolic engineering purposes (see for instance Cross et al., 2017; DeLoache et al., 2016; Dusséaux et al., 2020; Naseri, 2023). Using the *KanR* cassette flanked by long *ARG1* homology arms (Figure 4a) as a starting point, we subsequently inserted mCherry-PTS1 under control of the *S. stipitis GPD1* promoter into this cassette. The cassette was PCR amplified and transformed into NCYC102 cells. G418-resistant colonies were selected (Figure 4b), restreaked and grown into liquid media before imaging. As expected, a typical punctate fluorescent pattern was observed in the transformants (Figure 4c). Proper integration into the *ARG1* locus was confirmed by PCR (Figure 4d). We conclude the *ARG1* locus in NCYC102 cells provides a safe genome landing site for the expression of heterologous genes. Also, as NCYC102 has multiple copies of *ARG1*, the cells do not become arginine auxotrophs.

4 | CONCLUSION

Here, we describe an easy method for efficient gene targeting in *D. hansenii* isolates through homologous recombination using short flanks of only 50 bp. Although previous studies suggested that gene targeting through homologous recombination requires long regions of homology to target sites and occurs at low efficiency, we found this was not the case in our experiments as *ARG1* deletions in NCYC3363 and NCYC3981 together resulted in only one out of 22 transformants to be mistargeted. One likely explanation for this is that in previous studies auxotrophic markers were used. Since these markers are identical to the DNA sequences in the host genome, gene conversions may restore the auxotrophy, thereby reducing targeting efficiency as has previously been observed in *S. cerevisiae* (Wach et al., 1994). The high efficiency of targeted gene disruption prompted us to test whether short homology arms could stimulate gene targeting and indeed we found it could. However, we noticed a drop in the number of transformants, but through optimisation of the electroporation protocol, we now routinely obtain between 15 and 50 per electroporation using 500 ng PCR product. In line with our findings, double-strand breaks induced by CRISPR-Cas9 could be repaired by homologous recombination using 90 bp oligonucleotides in *D. hansenii* (Spasskaya et al., 2021), further illustrating that short homologous sequences are sufficient to mediate accurate homologous recombination in this yeast. Although there was no need for optimisation of homologous recombination and reduction of random integration in our experiments, there might be a need to do so when using certain natural isolates or difficult-to-target loci. For instance, the target DNA sequence of an isolate may slightly diverge from that of the homology arms of the PCR product. This is expected to affect the efficiency in homologous recombination and longer homology arms could be used. Alternatively, the DNA region around the target site could be sequenced in this isolate and the homology arms of the PCR product customised. Longer homology arms might also help in the creation of targeted genome modifications in isolates that are difficult to transform. If random integration is a problem, one could disrupt the nonhomologous end-joining pathway by deletion of *D. hansenii* Ku70 (Strucko et al., 2021).

One surprising observation was that the strain NCYC102 appears to have multiple copies of the *ARG1* gene. Most *D. hansenii* isolates are assumed to be haploid, but variations in genome size have been reported and some strains are diploid or even aneuploid (Jacques et al., 2010; Link et al., 2022; Petersen & Jespersen, 2004). Somatic ploidy variations, including aneuploidy, are not restricted to *D. hansenii* strains but are also found in, for instance, natural isolates of *S. cerevisiae* and *C. albicans*. Ploidy variations are considered to be adaptive responses to the environment and are reversible (for a

FIGURE 4 Strategy for heterologous expression through integration into the *ARG1* locus. (a) Schematic of the expression array of the KanR cassette followed by the mCherry-PTS1 expression unit, flanked by 0.9–1 kb homology regions to the *ARG1* locus to stimulate integration through homologous recombination. The CTG codon-adapted mCherry ORF was extended with a C-terminal peroxisomal targeting signal type I (PTS1) and expression is controlled by the *Scheffersomyces stipitis* GPD1 promoter region (Prom). Red arrow, direction of transcription. Black arrows containing numbers represent primers and the expected size of PCR products is indicated. Red and blue lines indicated upstream and downstream flanking regions of the *ARG1* gene locus, respectively. (B) Growth analysis of NCYC102 WT and two transformants on yeast medium (YMDeb) ± 500 µg/ml G418. (c) Epifluorescence microscopy image of NCYC102 cells transformed with mCherry-PTS1 that were grown for extended periods in the log phase. Image is a flattened *z*-stack. Cell circumference is labelled in blue. Scale bar: 5 µm. (d) Agarose gel electrophoresis analysis of PCR product as indicated in (a) on total DNA of wild-type cells (WT) or two G418-resistant colonies (1, 2) that are showing punctate mCherry labelling. M, molecular weight marker.

review see Todd et al., 2017). We have found several additional genes in this strain to still contain a WT copy after disruption of one copy, including *ADE2* and *FOX2* (not shown). However, we also found two genes to be single copy, suggesting that either the *ARG1*, *FOX2* and *ADE2* genes are duplicated or that NCYC102 is aneuploid. One possible explanation could be that the strain was diploid but is in the process of losing chromosomes over time to return to its haploid state. Whatever the underlying mechanism, we used the presence of multiple *ARG1* loci in NCYC102 to our advantage to generate a safe site for expression of heterologous genes. The use of PCR-based genome modifications is a very powerful tool for systematic approaches in the analysis of *D. hansenii* and the use of heterologous selectable markers allows to study not only the laboratory strain DH9 but also wild isolates.

AUTHOR CONTRIBUTIONS

Sondos Alhajouj, Tarad Abalkhail and Selva Turkolmez performed the *ARG1*, *ADE2* and mCherry-PTS1 genome modifications. Zeena Hadi Obaid Alwan and Sondos Alhajouj optimised electroporation. Ewald H. Hettema and Daniel James Gilmour conceived the original idea behind the project. Ewald H. Hettema, Sondos Alhajouj, Zeena Hadi Obaid Alwan, Tarad Abalkhail and Selva Turkolmez designed experiments. Ewald H. Hettema and Phil J. Mitchell supervised the research. Ewald H. Hettema made figures and wrote the manuscript with the input of all other authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Ewald H. Hettema http://orcid.org/0000-0003-2245-5287

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